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Simultaneous Binding of Heparin and Platelet Factor-4 to Platelets: Further Insights Into the Mechanism of Heparin-Induced Thrombocytopenia

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Heparin-induced thrombocytopenia (HIT) is mediated by antibody against complexes of platelet factor-4 (PF4) and heparin. Although it has been assumed that these complexes bind to platelets and provide a target for the antibody, this has never been demonstrated. Furthermore, there is evidence suggesting that heparin-PF4 complexes do not bind to platelets. We have analyzed the effect of each ligand on the platelet binding of the other. We particularly focused on the result when heparin and PF4 are in equimolar concentration because we had previously shown that this was the condition under which HIT-IgG increased on the platelet surface. We found that when the molar concentration of PF4 approximates or exceeds that of heparin, the ligands bind simultaneously to the cells and HIT-IgG binds also. However, when heparin is in molar excess, both PF4 binding and HIT-IgG binding are diminished. Our data are consistent with the hypothesis that heparin-PF4 complexes bind via their heparin component to heparin binding sites on the platelet membrane rather than by their PF4 component to PF4 sites. The conditions promoting the binding of the complexes also lead to binding of HIT-IgG. Am. J. Hematol. 58:24–30, 1998.

Key words: heparin; platelet factor 4; thrombocytopenia

INTRODUCTION

Platelet factor-4 (PF4) is a tetrameric protein (Mr 31,200) found in the alpha granules of platelets and notable for its extraordinary affinity for heparin [1]. These characteristics led investigators to suspect a role for PF4 in the pathogenesis of heparin-induced thrombocytopenia (HIT). In fact, the antibody mediating this disorder was shown to bind to microtiter wells coated with a mixture of heparin and PF4 [2–4]. The pathogenetic significance of this observation was supported by work from our laboratory demonstrating that IgG from patients with HIT binds to platelets when PF4 and heparin are present in approximately equimolar concentrations [5]. Exactly what happens to cause this binding, however, has remained unclear.

Because both heparin and PF4 are known to bind to platelets [6,7] and because PF4 and heparin form complexes with each other, [8,9], the assumption has been that heparin-PF4 complexes can also bind to the platelet and that these are the target of the autoantibody from patients with HIT [4,10,11]. Simultaneous binding of both ligands to platelets, however, has never been demonstrated. On the contrary, evidence has been reported to suggest that heparin-PF4 complexes may not be able to bind to platelets. It was shown, for example, that platelet-bound PF4 is displaced by heparin and that platelet releasate (rich in PF4) inhibits heparin binding to the cells [7,12].

To address these apparent discrepancies, we analyzed the effect of heparin on PF4 binding to platelets and of PF4 on heparin binding. We particularly focused on the binding of these ligands when they were present in approximately equimolar concentration since this is the condition that promotes platelet binding of IgG from patients with HIT (HIT-IgG) [5]. We chose to study activated platelets because our earlier studies had indicated that HIT-IgG binds more avidly to activated cells [5].

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Furthermore, by purposely causing the cells to secrete their PF4 and then separating them from their PF4-rich medium, we could minimize the concentration of endogenous PF4 in our platelet suspensions and thereby minimize the competition between the endogenous PF4 and the exogenous PF4 that we added in our experiments. We have previously shown that suspensions of platelets prepared in this way contain less than 6 nmol/L PF4 [5]. Since our studies were generally performed with concentrations of exogenous PF4 that were 5- to 100-fold greater than this, we assumed that the contributions of endogenous PF4 to our results were negligible.

MATERIALS AND METHODS

Heparin Preparations

A porcine heparin fraction of nominal Mr ~18,000 was obtained from Celsus Laboratories (Cincinnati, OH). By gel filtration through Superose-12 (Pharmacia Biotech, Uppsala, Sweden) in 0.5 M NaCl, we determined the median Mr to be ~10,000. Tritiated porcine heparin, [^{3}H(G)]-heparin, was purchased from SibTech, Inc. (Tenafly, NJ), mixed with unlabeled porcine heparin (Sigma Chemical Co., St. Louis, MO), and fractionated by gel filtration through Sephadex G200 (Pharmacia) in 0.5 M NaCl [13]. A portion of the leading part of the eluate was pooled. By repeat chromatography (Superose-12) the median Mr was determined to be ~12,000. The specific radioactivity of this fraction was 4.3 DPM/ng.

PF4 Preparation

PF4 was purified from outdated platelets using heparin-Sepharose (Pharmacia), as previously described, and for some applications was radiolabeled with ^{125}I (Amersham Corp., Arlington Heights, IL) using Iodo-Beads (Pierce Chemical Co., Rockford, IL) [5]. Because the solubility of PF4 is very limited at physiologic salt concentrations, we complexed the purified protein with glycosaminoglycan derived from platelet releasate, which includes the chondroitin sulfate that is naturally bound to platelets in vivo [14–16]. The source of this glycosaminoglycan was the fraction of platelet releasate that did not bind to heparin-Sepharose in 0.10 mol/L NaCl, 0.05 mol/L tris, pH 8. We quantitated this fraction in ''units,'' which we defined as the product of the volume of the fraction in mL and its absorbance at $\lambda = 280$ nm. This fraction was mixed with purified PF4 and dialyzed against 1.5 mol/L NaCl for 2 hr and then against distilled water overnight at 4°C. Whenever >40 μg of PF4 was incubated this way with 1 U of the glycosaminoglycan fraction, a fine precipitate was visible at the end of the dialysis, representing uncomplexed PF4 [15,16]. This was removed by ultrafiltration and the filtrate dialyzed into Buffer A (140 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L $NaHCO_3$, 0.5 mmol/L NaH_2PO_4, 4 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, 5.5 mmol/L glucose, pH 7.4). Aliquots of this were stored at −80°C until used.

By a chemical measurement of uronic acid content using glucuronic acid as a standard [17], we found that our preparations of the PF4-carrier complex contained 0.15 ng uronic acid per ng PF4. According to Barber et al., the uronic acid of this complex represents 32% of the weight of chondroitin sulfate chains with Mr 12,000 [15]. Therefore, we calculated that our preparations contained ~0.8 mol of PF4 per mol of chondroitin sulfate, which approximates the molar ratio of 1.0 reported by Barber et al. [15].

PF4 concentration was measured by one of two ELISAs, either the assay manufactured by Diagnostica Stago (Asnieres, France) or one from Behring (Marburg, Germany). In a preliminary study, we determined that the Diagnostica Stago assay gave results for the International Standard for PF4 (National Institute for Biological Standards and Control, Hertfordshire, England) that were 1.6 times the expected value, while the Behring assay gave results that were only 0.65 of expected [18]. Therefore, the concentration of PF4 measured in our preparations was adjusted by these factors as appropriate to conform to the International Standard.

Purification of HIT-IgG

IgG from a patient with well-documented HIT was purified using protein-G Sepharose (Pharmacia) and radiolabeled with ^{125}I using Iodo-Beads [5].

Platelet Preparation

Platelet-rich plasma containing 0.32% sodium citrate and 10 μmol/L prostaglandin E1 (Sigma) was separated from the whole blood of normal volunteers. The platelets were removed from the plasma by centrifugation through 10% arabinogalactan (Cellsep, Larex, Inc., St. Paul, MN) and resuspended in calcium- and magnesium-free Buffer A containing 10 mmol/L EDTA. The cells were then activated with thrombin (1 U/mL bovine thrombin for 5 min at room temperature) to release their PF4 and separated from their PF4-rich milieu by a second centrifugation through 10% arabinogalactan [5]. The cells were finally resuspended in calcium- and magnesium-free Buffer A (without EDTA) containing 1% bovine serum albumin and quantitated by a CELL-DYN 3500 (Abbott Laboratories, Abbott Park, IL). Final platelet concentration varied from ~4 × 10^8/mL to ~1 × 10^9/mL.

Platelet Binding Experiments

These were performed by mixing platelets suspended in Buffer A lacking divalent cations with equal volumes of ligand(s) dissolved in Buffer A containing 4 mmol/L Ca^{++} and 2 mmol/L Mg^{++}. In some experiments the final suspensions contained a constant concentration of PF4 (usually 100 nmol/L, either unlabeled PF4 or ^{125}I-PF4)

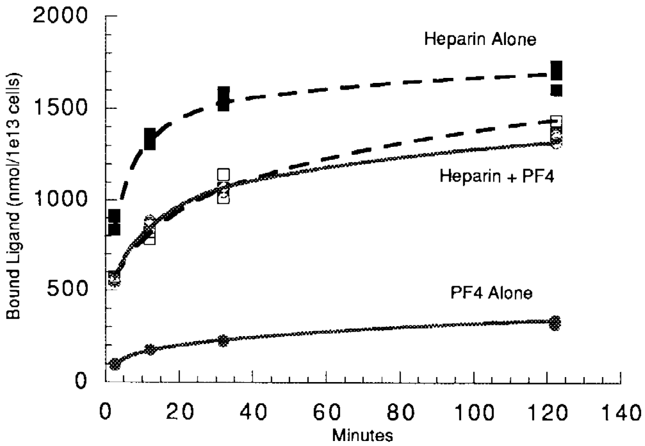

Fig. 1. Ligand binding over time. Platelets were suspended in 100 nmol/L heparin (squares, dashed lines) and/or 100 nmol/L PF4 (circles, solid lines) at time 0. The data points represent binding in three separate aliquots of suspension at the time points shown. When heparin and PF4 were mixed in equimolar concentrations, the data points were almost superimposable. The results are representative of five experiments.

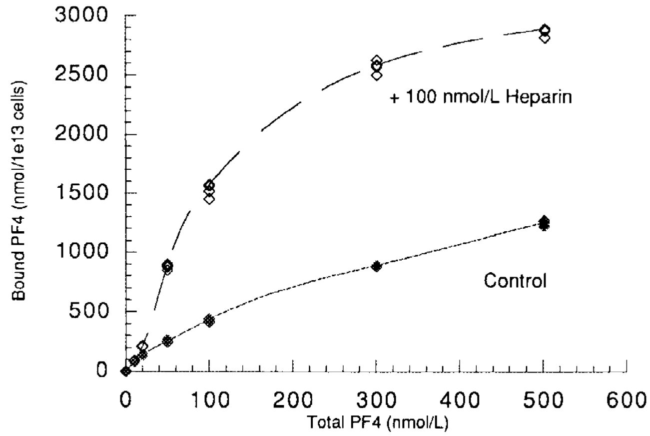

Fig. 2. Platelet binding of PF4 in the absence (control) or presence of constant 100 nmol/L heparin. Bound PF4 in 4 aliquots was measured for each of the total PF4 concentrations shown. The graph is representative of 7 experiments.

and variable concentrations of heparin (0–800 nmol/L, either unlabeled heparin or ^{3}H-heparin), whereas at other times the heparin concentration was kept constant (100 nmol/L) and the PF4 concentration was varied (0–600 nmol/L). Sometimes a constant concentration of 20 nmol/L ^{125}I-HIT-IgG was also included.

The mixtures were incubated at room temperature for 2 hr, which was shown to be adequate to reach equilibrium (Fig. 1). Following this the cells were recovered by centrifugation at 13,000*g* for 2 min. The cell pellets and aliquots of the supernatants were processed for gamma radiation counting or for liquid scintillation counting by standard methods. The measurements of radioactivity were converted to nmol of ligand bound per 10^{13} platelets or to nmol of ligand per liter.

RESULTS

Equilibration Time

When heparin was present alone, its binding to platelets reached equilibrium within 30 min, whereas equilibration of PF4 alone or mixtrues of heparin and PF4 required 1–2 hr (Fig. 1). Similarly binding of ^{125}I-HIT-IgG in the presence of equimolar PF4 and heparin reached equilibrium in 1–2 hr (not shown). Binding of ^{3}H-heparin and ^{125}I-PF4 was completely reversed by adding an excessive amount of unlabeled heparin.

Ligand Binding in the Presence of a Constant Concentration of Heparin and a Variable Concentration of PF4

As the concentration of PF4 was increased in platelet suspensions containing a constant amount of heparin, binding of PF4 greatly exceeded binding in the absence of heparin (Fig. 2). When PF4, heparin, and HIT-IgG were measured in the same experiment, the binding of all three ligands increased until the total molar concentrations of PF4 and heparin were approximately equal (Fig. 3). At that point, equimolar amounts of heparin and PF4 were also bound to the platelets and a peak of HIT-IgG binding occurred. At greater PF4 concentrations (i.e., with PF4 in molar excess), heparin binding remained stable or decreased slightly, whereas PF4 binding continued to increase and HIT-IgG binding decreased but never to its baseline value.

Addition of 1,000-fold excess unlabeled heparin virtually eliminated the baseline binding of ^{3}H-heparin. Addition of 200-fold excess unlabeled HIT-IgG only reduced baseline ^{125}I-HIT-IgG by ~30% (indicating that the majority of HIT-IgG binding in the presence of heparin alone is non-specific), whereas the peak of ^{125}I-IgG binding at equimolar heparin and PF4 was virtually eliminated (indicating that the peak represented IgG binding specific for the presence of heparin and PF4), as previously described [5].

Ligand Binding in the Presence of a Constant Concentration of PF4 and a Variable Concentration of Heparin

Compared with control platelets, platelets in a constant amount of PF4 bound approximately twice as much heparin when the concentrations of heparin and PF4 were equimolar (Fig. 4) but did not bind significantly different amounts of heparin at heparin concentrations of $\leqslant$0.5- or $\geqslant$2-fold the PF4 concentration. When measured in the same experiment, the binding of heparin, PF4, and HIT-IgG all increased together until heparin and PF4 were present in approximately equimolar amounts (Fig. 5). At this point, the amounts of platelet-bound heparin and PF4

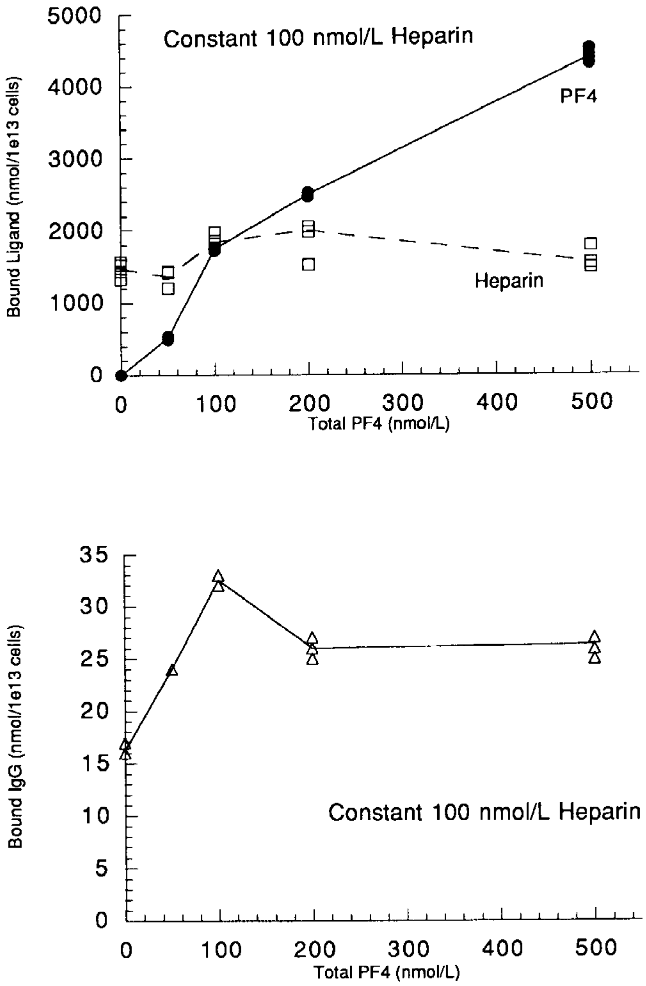

Fig. 3. Ligand binding in the presence of 100 nM heparin and a variable concentration of PF4. Platelets suspended in 100 nmol/L ^{3}H-heparin were mixed with PF4 (unlabeled when measuring bound ^{3}H-heparin or ^{125}I-IgG; ^{125}I-PF4 when measuring bound PF4) at the concentrations shown on the horizontal axes. The concentration of HIT-IgG (bottom) was 20 nmol/L. The data shown are results from a single study but are representative of 12 experiments. Three or 4 measurements were made at each PF4 concentration.

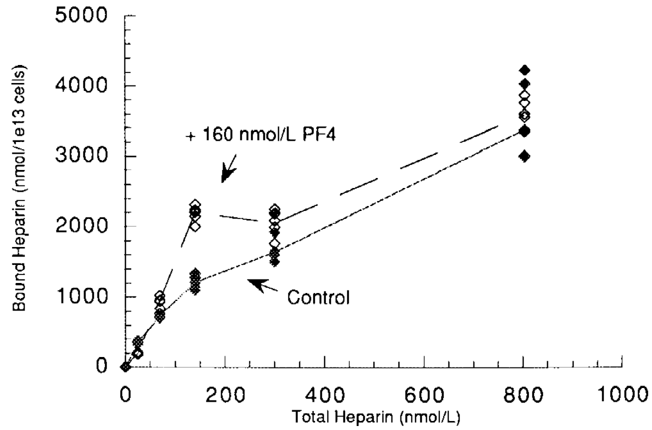

Fig. 4. ^{3}H-heparin binding to platelets in the absence (control; solid triangles, solid line) and presence (open triangles, dashed line) of 160 nmol/L PF4. Four measurements were made at each total heparin concentration. The data are from a single experiment but are representative of 7 experiments.

were also equimolar. At greater heparin concentrations (i.e., with heparin in molar excess), HIT-IgG decreased to baseline and PF4 fell below its baseline value. We have previously shown that in such experiments the addition of a 200-fold excess of unlabeled HIT-IgG (from the same stock as the ^{125}I-HIT-IgG) reduced baseline binding by ~30% and eliminated the increased ^{125}I-HIT-IgG binding observed when heparin and PF4 are equimolar [5].

DISCUSSION

The in vitro evidence is convincing that HIT-IgG binds to complexes of heparin and PF4 and that HIT-IgG also binds to platelets that are suspended in equimolar concentrations of heparin and PF4 [2–5]. However, the spacial arrangement of these molecules on the platelet surface has remained unclear. There has been speculation that complexes of heparin, PF4, and IgG are attached to platelets at binding sites for PF4 [4,11]. However, because heparin displaces PF4 from platelets, the question is raised of how these ligands can provide a target for HIT-IgG on the platelet membrane [7]. On the other hand, there is also evidence that the complexes bind directly to FcγRII receptors on the cells [3], although earlier work from our laboratory indicated that F(ab′)$_2$ fragments of HIT-IgG can also bind [5].

We have now demonstrated that heparin and PF4 can bind simultaneously to platelets, independently of HIT-IgG, but only when the molar concentration of PF4 approximates or exceeds the molar concentration of heparin. Because these are the conditions that also promote platelet binding of HIT-IgG, we believe that HIT-IgG is bound (via its F(ab′)$_2$ terminus) to heparin-PF4 complexes that are, in turn, bound to the platelet membrane (Fig. 6).

Although we have not directly shown that heparin and PF4 bind to platelets in a complex, this seems highly likely because of the extraordinary affinity of the two molecules for each other [1] and because each ligand affects the binding of the other. The effect of the molar ratio of PF4 to heparin on the binding of these ligands to platelets is apparent in experiments in which one ligand is held constant while the other is varied. If heparin is kept at 100 nmol/L, for example, while PF4 is increased, the amount of PF4 bound to the platelets greatly exceeds that bound to the control cells (Fig. 2), except at the lowest concentrations of PF4. In contrast, if PF4 is held constant while heparin is added, heparin binding to plate-

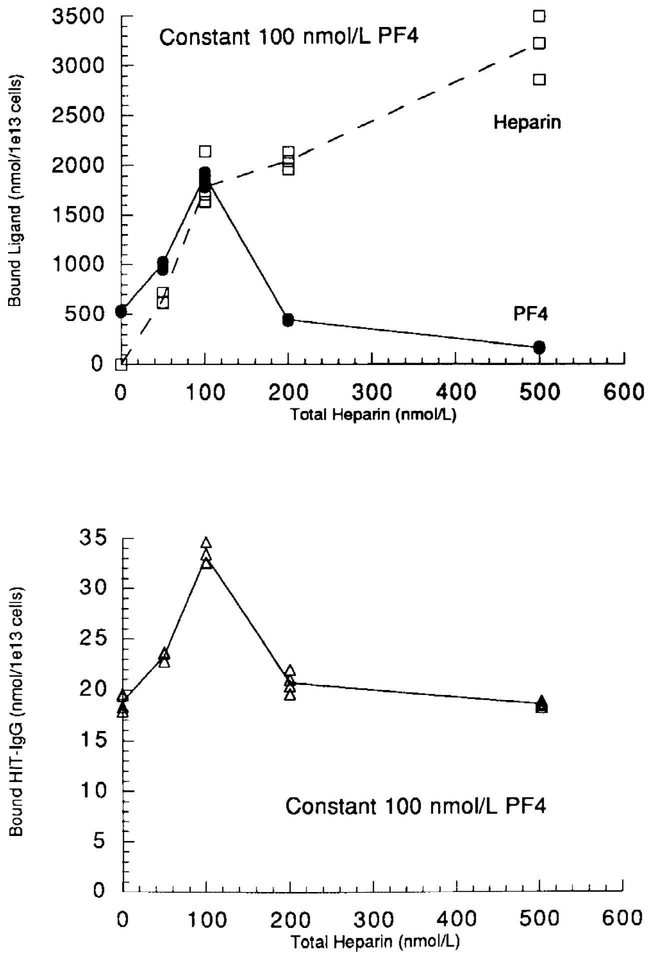

Fig. 5. Ligand binding to platelets in the presence of 100 nmol/L PF4 and a variable concentration of heparin. Platelets suspended in 100 nmol/L PF4 (unlabeled when measuring bound heparin or ^{125}I-PF4 when measuring bound PF4) were mixed with ^{3}H-heparin in the concentrations shown. Some cells were suspended in 100 nmol/L PF4 (unlabeled) and 20 nmol/L ^{125}I-HIT-IgG (bottom) before being mixed with heparin. The data shown are from a single study but are typical of eight experiments.

lets exceeds the control when the molar concentration of PF4 approaches the concentration of heparin (Fig. 4). As heparin concentration becomes excessive, however, heparin binding in the presence of PF4 becomes indistinguishable from the control.

The interactions underlying this behavior are suggested by experiments in which heparin and PF4 on the platelet surface are both measured while one ligand is held constant and the other is varied. The results are consistent with a hypothesis depicted in cartoon form in Figure 6. The key feature of this hypothesis is that *heparin-PF4 complexes bind to heparin-binding sites by their heparin component rather than to PF4-binding sites by their PF4 component.* This idea was also suggested by a previous report that immune complexes related to HIT attach to the platelet membrane via sulfated oligosaccharides [10].

When PF4 is held constant, for example, while heparin is increased, not only does heparin binding proceed, but PF4 binding increases (Fig. 5, top). When the total concentrations of heparin and PF4 in the suspension are equimolar, the amounts of platelet-bound heparin and PF4 are also equimolar, and there is an increase in the amount of cell-bound HIT-IgG (Fig. 5, bottom). As heparin concentration surpasses PF4 concentration, however, PF4 binding decreases, and HIT-IgG binding returns to its baseline level. According to our model, this behavior is explained by the binding of heparin-PF4 and heparin-PF4-HIT-IgG complexes at heparin binding sites (Fig. 6A,B). As uncomplexed heparin increases, it competes with the complexes and effectively displaces them from the heparin sites (Fig. 6C).

A different pattern is observed in the converse experiment, in which heparin concentration is constant and PF4 is varied. When heparin is in molar excess, the binding of heparin-PF4 complexes is blunted by competition from uncomplexed heparin to produce a slightly sigmoid binding curve (Fig. 2; Fig. 3, top; Fig. 6D). When the molar concentration of PF4 equals or exceeds that of heparin (Fig. 6B,E), PF4 binding is greater than it is in the absence of heparin (Fig. 2), while heparin binding increases only slightly if at all (Fig. 3, top; Fig. 6E).

Therefore, when PF4 is in excess, heparin binding persists in the form of heparin-PF4 complexes at heparin-binding sites (Fig. 6A,E), whereas when heparin is in excess, PF4 exists in heparin-PF4 complexes that are displaced from the cells by uncomplexed heparin (Fig. 6C,D). HIT-IgG binding is maximal when equimolar amounts of heparin and PF4 are present on the platelets (Figs. 3 and 5, bottom). If the molar concentration of PF4 falls below that of heparin, HIT-IgG binding decreases in parallel with bound PF4 (Fig. 5). However, if the molar concentration of PF4 surpasses that of heparin, HIT-IgG binding decreases but persists, implying a lower affinity of the antibody for larger complexes with excess PF4 (Fig. 3, bottom).

These observations suggest why many patients who develop HIT-IgG do not become thrombocytopenic [19–23]. While heparin-PF4 complexes appear to be remarkably immunogenic, we postulate that the appearance of HIT-IgG is clinically inconsequential unless the molar concentration of PF4 in the circulation approximates or exceeds that of heparin. This circumstance must be uncommon in clinical practice since the therapeutic range for heparin is 0.2–0.4 U/mL, corresponding to ~100–200 nmol/L. For PF4 to reach this concentration would require extensive platelet activation [24]. Because heparin is typically present in sufficient excess to prevent heparin-PF4 complexes from binding to platelets, HIT-IgG does not bind and platelet destruction is avoided. If the

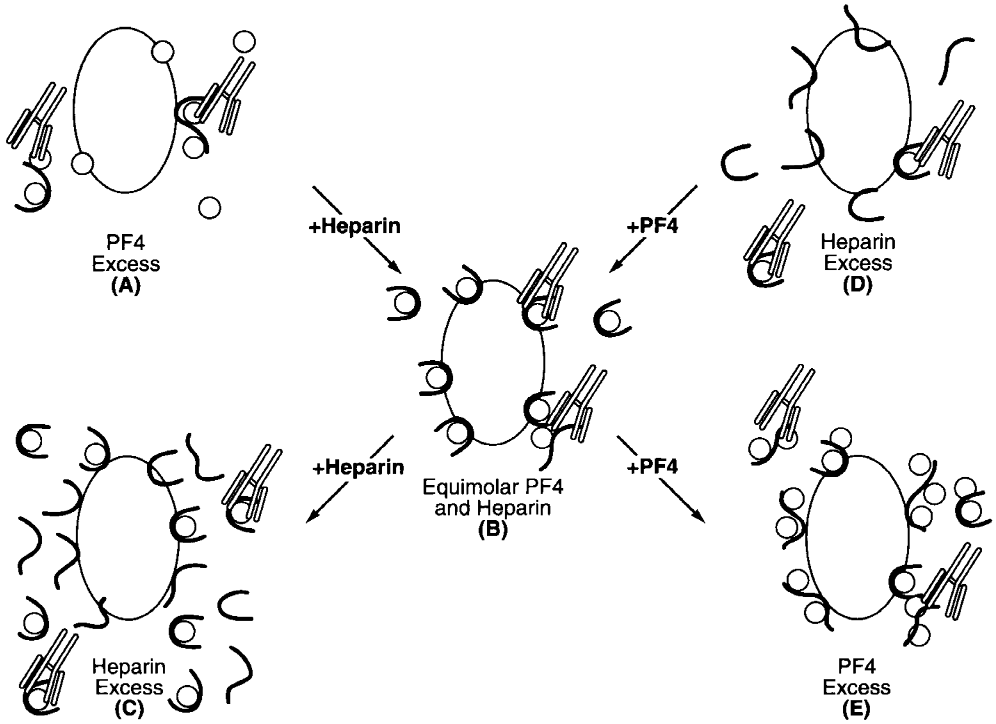

Fig. 6. Cartoon depicting the hypothesis consistent with the platelet binding studies. PF4 is represented by the small open circles and heparin by the black, polymorphic lines. HIT-IgG is shown as stick figures. A,B, and C contain the same number of PF4 molecules, and D,B, and E the same number of heparin molecules.

molar ratio of PF4 to heparin approximates or exceeds unity, however, heparin-PF4 complexes bind to the platelet membrane and provide targets for HIT-IgG. HIT-IgG binding then stimulates the cells and leads to their removal.

If this reasoning is correct, patients whose clinical situations are less associated with platelet activation and high levels of circulating PF4 are not only at lower risk for developing HIT-IgG, but are also relatively safe from the adverse effects of this IgG if it should appear. On the other hand, clinical events associated with platelet activation, such as recent surgery, will lead to a higher incidence of HIT-IgG and a greater risk of clinical consequences resulting from it. Indeed, emerging clinical data are consistent with this hypothesis [20–23,25].

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